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**JOINT PROGRESS REPORT**

Period: Feb 15, 2010 – March 1, 2011

Proposal Number: **BC084220** (PI: Carvalho) and **BC084220 P1** (PI: Frangos)
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## INTRODUCTION

Cancer tissues usually present high interstitial fluid pressures (IFP) which reduce the transport of therapeutic agents by decreasing convection from blood into cancer tissues, increasing the possibility of poor treatment outcome in breast cancer [1]. The larger the molecular weight of the drug, the higher the detrimental effect of interstitial hypertension on drug delivery [2]. Several factors affect the convective transport of drugs across the vascular wall, which can be described by the Starling-Landis equation [3]. These factors include the IFP, the capillary hydrostatic pressure and the capillary and interstitial fluid (IF) colloid osmotic pressure, among others. Microgravity (or simulated gravity) exposure significantly increases the capillary osmotic pressure, which in turn compensate for increased IFP and improves the net transcapillary convection of drugs. We hypothesize that simulated gravity will improve the convection of nano-particles in breast cancer, therefore improving drug delivery.

We will address this issue by submitting mice with implanted breast tumors of human or mouse origin to simulated microgravity and measuring a number of parameters in the Starling-Landis equation, including IFP, capillary hydrostactic pressure and the capillary and IF colloid osmotic pressure. In addition, we will measure under the same conditions the convective transport of nano-particles by using dextrans of different molecular weights labeled with fluorochromes. The data obtained will provide evidence as to whether simulated microgravity improves drug convection to cancer tissues and therefore can be considered as a tool in the fight against cancer.

## BODY

In the report from last year, we described several factors that went into standardizing procedures for measurement of tumor interstitial fluid pressure, establishing conditions for measuring the convection of fluorochrome-labeled dextrans to tumor tissue, and establishing a reliable method to collect interstitial fluid (IF) sample from tumors. Since February of 2010, we have completed additional experiments to finalize the details of our procedure methods as it pertains to **Task 3** from the revised statement of work, establishing the breast cancer implantation, and conducted many simulated microgravity experiments related to **specific aim 2** described in the original grant, describing the effects of microgravity simulation on the convection of nano-particles in breast cancer. In this time period, we have made significant strides and have learned that our initial hypothesis requires additional details to completely understand the effects of microgravity simulation on the convection of nano-particles in breast cancer.

Our original hypothesis stated that microgravity simulation by head-down tilt will increase drug uptake in breast cancer when the tumor is located *below* heart level during head-down tilt, such as in tumor metastasis to the brain or eye. Our experiments, however, indicate that drug delivery in breast cancer is increased only when the tumor is located *above* heart level during head-down tilt

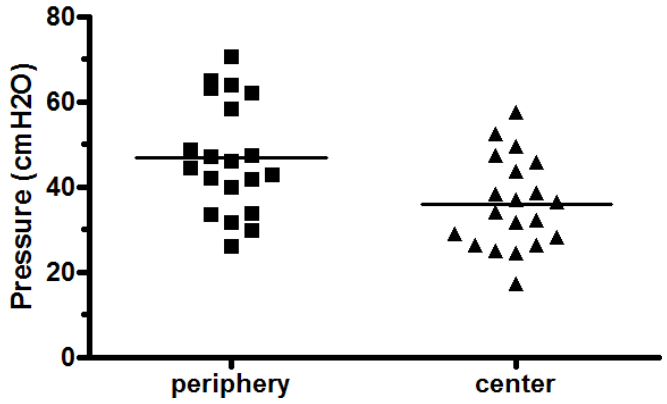
**Task 3: Establishment of the breast cancer implantation as it pertains to establishing conditions for measuring the convection of fluorochrome-labeled dextrans to the tumor tissue**

**1.** *Differences in peripheral and central pressure measurements of tumor masses*

In last year's report, we described differences between the core and periphery of tumors. We noted that the core is highly vascularized and is susceptible to necrosis, whereas the periphery has fresh, densely packed cells. We further explored these differences in measuring tumor interstitial fluid pressure (tumor IFP) in the periphery and center of the tumor mass.

For our experiments, mice were implanted with PY8119 cancer cells. When tumors reached 10mm in diameter, animals were anesthetized and tumor IFP in the periphery was measured. The periphery was determined by introducing the pressure transducer only 2mm (the length of the transducer) into the cancer tissue. In the same tumor, the pressure transducer was then inserted further to the center of the tumor. The center was determined by introducing the pressure transducer half the distance of the longest length of the tumor (measured using calipers prior to anesthetizing the mouse). Our results showed that pressure measurements in the periphery of the tumor mass were consistently higher than those at the center ($p$ = 0.0124, **Figure 1**).

**Figure 1.** Tumor IFP in the periphery and center of breast cancer tumors.




**2.** *Fluorescence detection of fluorochrome-labeled dextrans in tumor tissue*

At the time of completion of last year's report, we proposed to use different fluorochromes for each dextran size to detect convection of each dextran in the same animal. The advantage to this strategy was to decrease the total number of animals in the experiments. Suitability of this approach was shown by presenting data that showed there is no interference in the emission spectra of each dextran in a mixed solution. However, additional experiments showed that there are high levels of background emission for both tumor IF and plasma from a mouse that had not been injected with dextrans that correspond with the emission wavelength for three of the four dextrans (Cascade Blue, TMR, and TxR, **Table 1**). These high levels of background emission interfere with experimental data and render results with these dyes useless. Only FITC-labeled dextran dye showed consistently low levels of background emission and proved to be suitable for our experiments. Therefore, the original approach for measuring convection of nano-particles to

tumor tissue using different sizes of FITC labeled dextran dye outlined in the grant has been used.

**Table 1.** Background emissions for tumor IF and plasma from a mouse that had not been injected with dextran dyes.

| **Wavelength = 400nm Corresponding to Cascade Blue** | | **Wavelength = 494nm Corresponding to FITC** | | **Wavelength = 555nm Corresponding to TMR** | | **Wavelength = 595nm Corresponding to TxR** | |
|---|---|---|---|---|---|---|---|
| **Tumor IF** | **Plasma** | **Tumor IF** | **Plasma** | **Tumor IF** | **Plasma** | **Tumor IF** | **Plasma** |
| **148** | 125 | 15 | 13 | 265 | 346 | 192 | 230 |
| **177** | 112 | 37 | 4 | 355 | 324 | 243 | 217 |
| | | 10 | 4 | 312 | 312 | 241 | 229 |
| | | 16 | 6 | 304 | 330 | 286 | 227 |
| | | | | 294 | 329 | 233 | 228 |
| | | | | 351 | 340 | 252 | 232 |

**3.** *Hemoglobin quenching of FITC-labeled dextran dye*

As experiments progressed, we discovered that the potential presence of hemoglobin from mouse plasma samples interferes with FITC-labeled dextran dye signal. Using plasma from control mice enrolled in previous experiments, we measured hemoglobin content (Hb) against fluorescence signal of 40 and 3kD FITC-labeled dextran dye.

For 40kD FITC-labeled dextran, there was a 16.1% decrease in signal at 0.2 g/dL of Hb and no effect on signal at 0.1 g/dL of Hb (**Figure 2**). With 3kD FITC-labeled dextran, there was a 27.0% decrease in signal at 0.25 g/dL of Hb and 7.5% decrease in signal with 0.125 g/dL of Hb (**Figure 3**). To make such interference negligible in our experiments, Hb quenching was counter-acted by diluting samples 10-fold in saline and setting a cutoff of 0.15 g/dL Hb.

**Figure 2.** Hemoglobin quenching of 40kD FITC-labeled dextran dye.

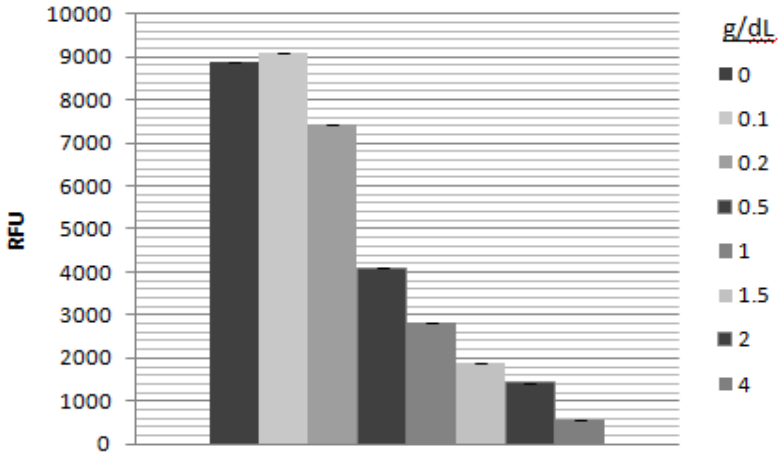
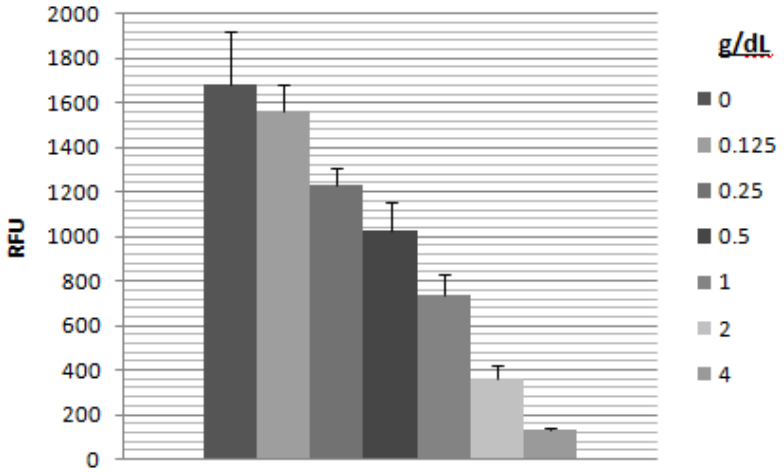



**Figure 3.** Hemoglobin quenching of 3kD FITC-labeled dextran.

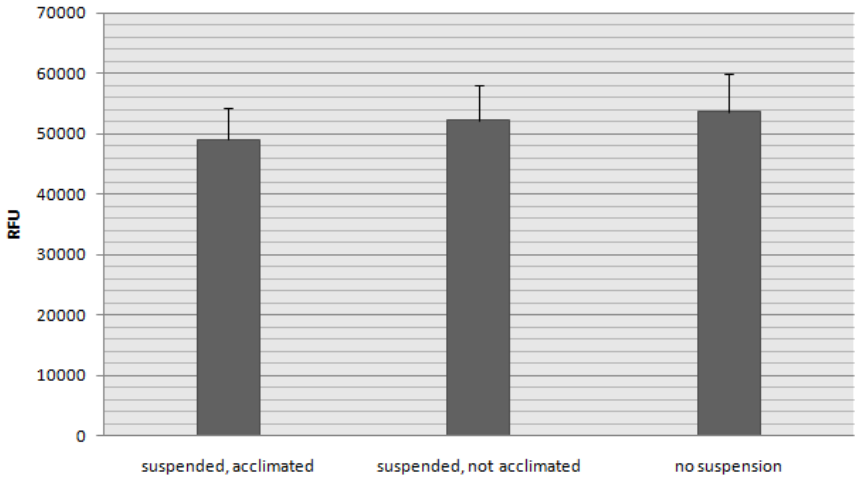



**4.** *Effects of suspension in plasma fluorescence for acclimated mice*

In addition, we conducted an experiment to determine the effects of stress on the mice in plasma fluorescence. One group of mice was suspended for 20 minutes a day for 4 days. On the fifth day, the same group was injected with 40kD FITC-labeled dextran intravenously and underwent tail suspension for 20 minutes. Another group also underwent tail suspension for 20 minutes but was not acclimated. A third group did not undergo suspension, serving as controls. After 20 minutes, maximum volume of blood was collected via cardiac puncture and tumor IF was collected via the wet wick method. Hemoglobin was measured for all samples and any sample that exceeded the above described cutoff of 0.15g/dL Hb content was discarded. Remaining samples were diluted 1:10 in saline and read in a spectrophotometer (Spectramax, Molecular Devices Inc.).

Our results showed that acclimation does not have an effect on plasma fluorescence data (p = 0.548, **Figure 4**). Therefore, we did not incorporate an acclimation period in our protocol.

**Figure 4.** Effects of acclimation on plasma fluorescence data in suspended mice.




## REPORTABLE OUTCOMES

We have focused our efforts on nano-particle delivery to determine effects of microgravity simulation by head-down tilt in breast cancer (**specific aim 2**).

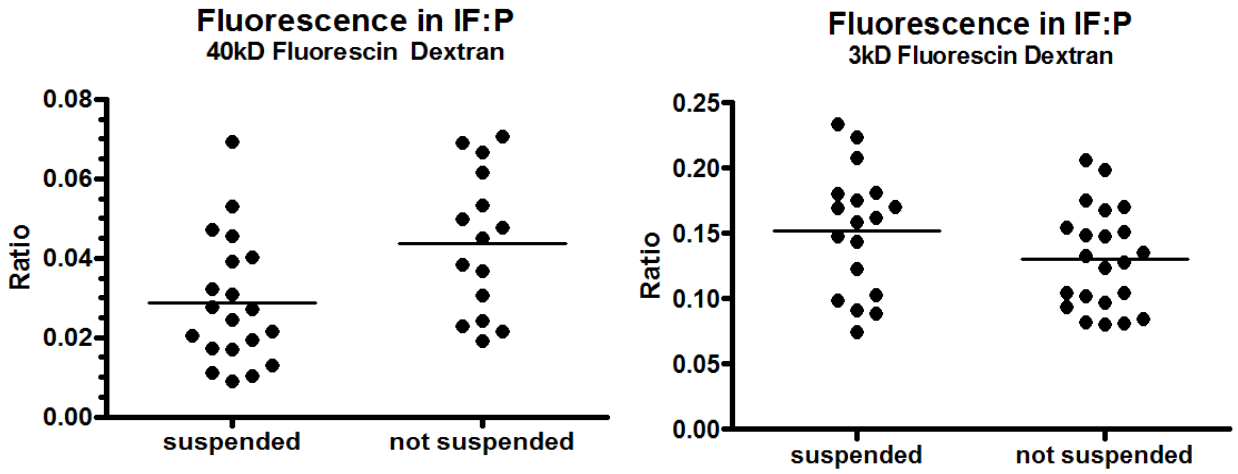
### Specific Aim 2: Effects of microgravity simulation on the convection of nano-particles in breast cancer

**1.** *Effects of tail suspension on tumor uptake of 40 and 3kD FITC-labeled dextran dye*

We evaluated the uptake of 40 and 3kD FITC-labeled dextrans in mice undergoing simulated microgravity. Two experimental groups were implanted with PY8119 tumor cells in the pectoral mammary fat pad. One group underwent tail suspension for 4 hours and the other did not, serving as controls. After this period, FITC-labeled dextran was injected intravenously and allowed to circulate for 20 minutes. After this period, samples were collected and analyzed as described in the prior section. Fluorescence data was normalized by comparing the ratio of tumor IF to plasma fluorescence (IF:P).

There was a significant reduction in IF:P signal ratio of mice injected with 40kD fluorescein dextran relative to controls ($p$ = 0.0206, **Figure 5**). There was no difference in IF:P signal ratio of mice injected with 3kD fluorescein dextran relative to controls ($p$ = 0.154, **Figure 6**).

**Figure 5 and 6.** Effects of 4 hour tail suspension on IF:P of 40 and 3kD FITC-labeled dextran dye.



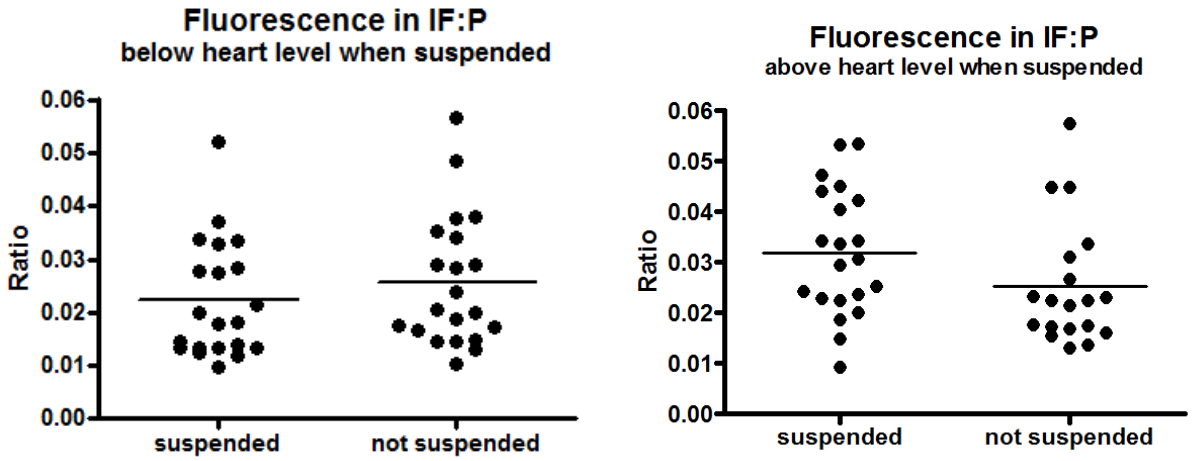

**2.** *Effects of tail suspension on tumor uptake based on location of tumor*

The above data suggests that hind limb suspension might decrease, rather than increase, nano-particle uptake by the tumor tissue. However, our consultants raised a concern that the close proximity of the tumor implanted in the pectoral mammary fat pad would minimize the effect of hind limb suspension while affecting other tissues with confounding effects. We decided, then, to evaluate the effects of hind limb suspension on dextran delivery to tumor tissue located further below heart level as well as above heart level.

Two groups of mice were implanted with PY8119 tumor cells in the submandibular fat pad (in the neck region) and two groups were implanted with the same cells in the inguinal fat pad (near the thigh). Each group was injected with 40kD FITC-labeled dextran intravenously. For each tumor location, one group underwent tail suspension for 20 minutes and the other did not, serving as controls. After this period, samples were collected and analyzed as described above.

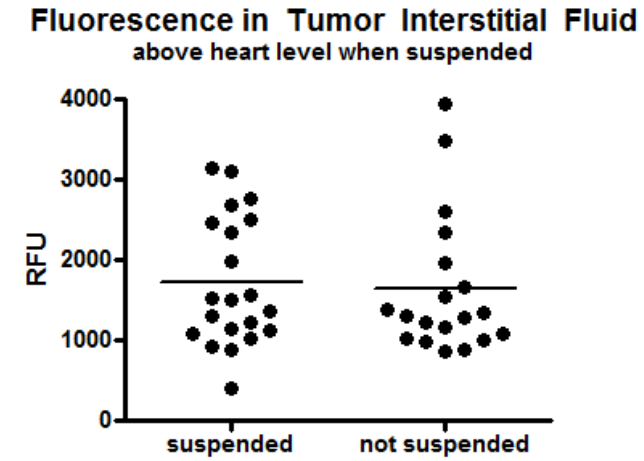
There was no statistical significance in IF:P signal ratio for tumors implanted below heart level ($p = 0.1996$, **Figure 7**). However, we found a statistically significant increase in dextran delivery when the tumor was located above heart level ($p = 0.0466$, **Figure 8**).

**Figure 7 and 8.** Effects of 20 minute tail suspension on IF:P based on tumor location.




Interestingly, suspension when tumors are located above heart level did not have an effect on fluorescence in tumor interstitial fluid but instead decreased dextran fluorescence in the plasma (**Figures 9 and 10**), showing that suspension has a more systemic effect on tissue distribution and perhaps on clearance of nano-particles. More importantly, when tumors are located below heart level, this decrease in plasma fluorescence was paralleled by a decrease in interstitial fluid fluorescence (**Figures 11 and 12**). These findings suggest that our initial hypothesis, that microgravity effects nano-particles drug delivery to the tumor tissue, may be correct but with a reverse effect. That is, the effect was observed in tissues located above heart level, not below heart level as originally proposed. Our next step is to define whether this effect is related to changes in tumor interstitial fluid pressure. We also find it relevant to verify that hind limb suspension affects the efficacy of anti-tumor drugs such as doxorubicin.

**Figure 9 and 10.** Effects of 20 minute tail suspension on fluorescence in tumor interstitial fluid and plasma when tumor is above heart level during suspension.



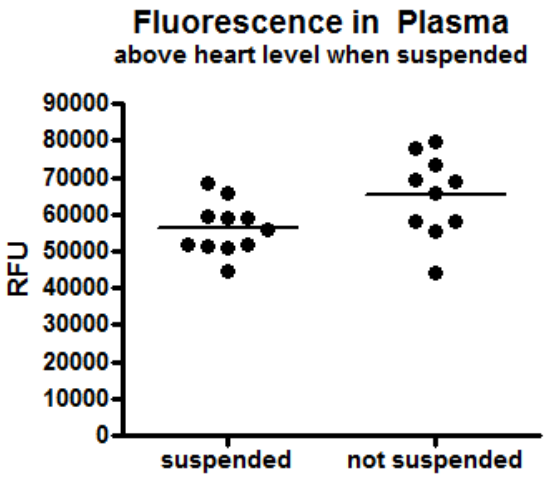
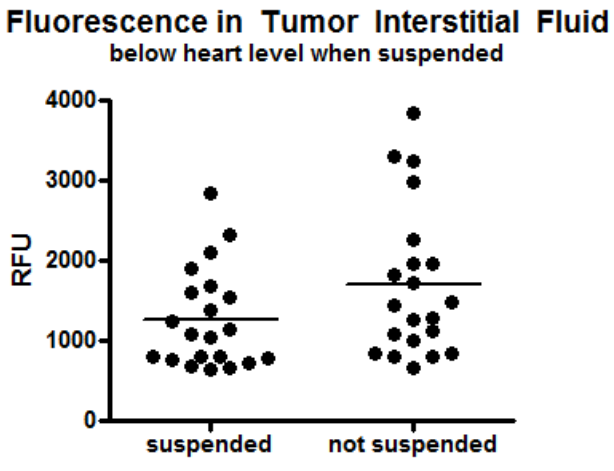
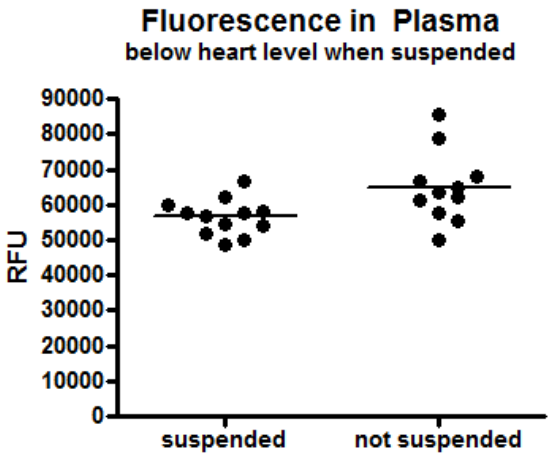




**Figure 11 and 12.** Effects of 20 minute tail suspension on fluorescence in tumor interstitial fluid and plasma when tumor is below heart level during suspension.




**CONCLUSION**

Effects of suspension when the tumor is implanted in the pectoral mammary fat pad (at heart level) decreased drug delivery to the tumor. Furthermore, drug delivery to the tumor increased when the tumor is implanted above heart level. These results indicate that, in the mouse, simulated microgravity increases nano-drug delivery to tumor tissues located above heart level.

## KEY RESEARCH ACCOMPLISHMENTS

The most important research accomplishment was the definition that simulated microgravity indeed has a positive effect in increasing nanoparticle uptake by the tumor tissue. However, contrary to our initial expectations, the beneficial effect was observed on tumors located above heart level during suspension. In tumors located at, or below, heart level when suspended the effect was not significant or even led to a decrease in dextran uptake by the tumor. Although more research is needed to better understand these findings, there is an indication that simulated microgravity may improve drug delivery to tumors located above heart level.

Other important accomplishments were the technical developments achieved during the development of this study which may be relevant to future studies, such as the definition of proper dyes, the interference of hemoglobin in the measurements, the effects of tumor location, the timing for suspension and dye injection, and the effect of acclimation on the measurements.

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## APPENDICES

None.